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**IMMUNE FUNCTION AND THE DECISION TO DEPLOY WEAPONS DURING FIGHTS IN THE
BEADLET ANEMONE *ACTINIA EQUINA***

Running title: Immunity and weapon use during fights

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Summary statement

Here, we demonstrate how strategic fighting decisions affect an individual's subsequent ability to mount an immune response.

Abstract

The ability to mitigate the costs of engaging in a fight will depend on an individual's physiological state. However, the experience of fighting itself may in turn affect an individual's state, especially if the fight results in injury. Previous studies found a correlation between immune state and fighting success, but the causal direction of this relationship remains unclear. Does immune state determine fighting success? Or does fighting itself influence subsequent immune state? Using the beadlet anemone *Actinia equina*, we disentangle the cause and effect of this relationship, measuring immune response once pre-fight and twice post-fight. Contrary to previous findings, pre-fight immune response did not predict fighting success, but rather predicted whether an individual used its weapons during the fight. Furthermore, weapon use and contest outcome significantly affected post-fight immune response. Individuals that used their weapons maintained a stable immune response following the fight, while those that fought non-injurious did not. Furthermore, although winners suffered a similar reduction in immune response to losers immediately post-fight, winners began to recover pre-fight levels within 24 hours. Our findings indicate that immune state can influence strategic fighting decisions and moreover that fight outcome and the agonistic behaviours expressed can significantly affect subsequent immunity.

Keywords: *Actinia equina*, contest behaviour, fighting success, immune response, injuries, weapon use

51

52 **1. Introduction**

53 The costs of engaging in a fight can be substantial, and in fact often outweigh the potential
54 benefits. It is for this very reason, that the majority of fights are settled using non-injurious
55 behaviours such as agonistic displays. But even non-injurious behaviours can be
56 physiologically costly to produce, for example leg displays used by the sierra dome spider
57 *Neriene litigiosa* require 3.5x more energy than resting (Decarvalho et al., 2004), while ‘trials
58 of strength’ such as wrestling in the house cricket *Acheta domesticus* demand 40x more
59 oxygen than a resting state (Hack, 1997). Furthermore, when fights cannot be settled via
60 non-injurious means, opponents escalate into the use of agonistic attacks in an effort to
61 force their opponent to retreat. Attacks can result in injuries to the recipient and, as
62 recently reviewed, to the attacker itself (Lane and Briffa, 2017). As animals have finite
63 energy stores, participation in a contest could reduce capacities for general maintenance,
64 including the ability to mount an efficient immune response.

65 The ability to maintain a normal immune system and mount an adequate reaction to
66 infection requires a great deal of resources. Even a mild infection can bring about huge
67 shifts in resource allocation, increasing both nutritional and metabolic demands (Lochmiller
68 and Deerenberg, 2000). It is thus generally assumed that only individuals of high quality are
69 capable of maintaining a normal immune system while also investing in other costly traits.
70 As a result, costly sexually selected traits including elaborate ornaments, courtship
71 behaviours (Folstad and Karter, 1992; Gilbert and Uetz, 2016) and even sperm quality
72 (Simmons, 2011) can provide an honest signal of an individual’s condition to potential mates
73 (i.e. the immunocompetence handicap hypothesis (e.g. Folstad and Karter, 1992; Gilbert and
74 Uetz, 2016; Simmons, 2011)). In addition to sexual signals, immunity has also been found to
75 covary with fighting ability and social status. However, despite recently explained
76 similarities between courtship and fighting (Mowles and Ord, 2012), the links between
77 immunity and agonistic behaviour have yet to be resolved. Previous work has indicated that
78 in general, immunity is positively correlated with fighting success (although see Vaananen et
79 al., 2006 for an exception), with winners exhibiting higher general immunity (Dijkstra et al.,
80 2011; Filby et al., 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012)

and an increased ability to respond to infection in comparison to losers (Hawley et al., 2007; Kelly, 2014; Zuk and Johnsen, 2000). These findings suggest that immunity may be a predictor of fighting success, such that individuals possessing higher immune abilities are better able to win fights. However, in all of these studies, immunity was only measured once, either before or after a fight and thus the evidence gathered to date does not allow us to disentangle the causal direction of the relationship between immunity and fighting success. Does pre-fight immunity predict fighting success or does fighting success affect subsequent immunity?

Fighting may affect immunity for several different reasons. Firstly, as mentioned earlier, fighting is energetically costly and may trade-off with immunity over resource allocation. Secondly, exposure to stress can cause changes in immune response (Yin et al., 1995; Demers and Bayne, 1997; Ortuño et al., 2001; Padgett and Glaser, 2003) and thus entering into a direct contest with another individual may modulate an individual's immune response regardless of the behaviours expressed within the contest or indeed the outcome of the interaction. Finally, if a contest escalates into the use of agonistic attacks, injuries may result for either one or both of the contestants, as a result of injuries inflicted by the opponent and of damage caused by an individual's own agonistic behaviour (see Lane and Briffa, 2017). Injuries not only require the reallocation of energy and resources in order to heal, but may also increase an individual's susceptibility to pathogens, especially when the injury incurred is an open wound. Injuries thus require an acute upregulation of immune response in order to both protect an individual from infection and to heal the wound (Lochmiller and Deerenberg, 2000). Consequently, changes in immunity triggered by a particular fight may depend upon the level of agonistic behaviour expressed by each opponent as well as the outcome of the fight.

Here, we explore the effects of fighting behaviour on the immune response of the beadlet sea anemone *Actinia equina*. *A. equina* are one of the simplest organisms to engage in contests. *A. equina* possess weapons in the form of specialised stinging structures called acrorhagi which are full of stinging cells (nematocytes) and used solely to attack conspecifics (Williams, 1978; Brace et al., 1979; Bigger, 1982). *A. equina* provide an interesting system for studying immune response in relation to fighting as they incur injury not only by receiving attacks but also by inflicting attacks (Lane and Briffa, 2017; also see figure 1).

During an attack, pieces of the attacker's acrorhagial epithelium peel off and stick to the recipient. This act causes localised necrosis on the recipient, but also has the unavoidable effect of ripping holes in the acrorhagi of the attacker, such that both individuals may be susceptible to infection. It has recently been demonstrated that the mucus produced by *A. equina* as a protective coating against desiccation and other environmental stressors contains antimicrobial properties (Stabili et al., 2015). One such property is a lysozyme-like enzyme which acts as an antibacterial agent, degrading the cell walls of gram-positive bacteria such as *Micrococcus lysodeikticus*. Lysozyme is one of the best characterised enzymes in self-defence against bacteria and has previously been shown to play a key role in controlling the inflammation and development of subcutaneous infection, for example in mice (Ganz et al., 2003). Here, in order to examine the effects of fighting and injury (including self-inflicted injuries) on immune response in *A. equina*, we measure lysozyme-like activity at three different time points, once before a fight and twice afterwards. We then calculate the change in lysozyme-like activity over the three measurements with respect to both fight outcome and injury state. Thus, we quantify the cost of self-inflicted injuries for the first time in any fighting animal. A comparison of post-fight lysozyme activities between individuals that inflict and receive epithelial peels will reveal the relative costs of injuries received from an opponent and injuries that are self-inflicted. If inflicting peels is costly in terms of immune response, individuals in poor condition should be less likely to use their weapons to inflict peels compared with those in good condition. In this case, pre-fight immune status should predict the chance of using acrorhagi in a subsequent fight. Fundamental contest theory assumes that for dangerous fighting to be adaptive, the benefits of any agonistic behaviour must outweigh the costs but this assumption has yet to be tested in terms of self-inflicted injuries.

2. Materials and methods

(a) Anemone collection and staging contests

Actinia equina (N=97) were collected intertidally from Portwrinkle (Cornwall UK; grid reference: SX 357539) between November 2016 and June 2017 and taken back to the lab within 1-2 hours of collection. As in previous studies of agonistic interactions in *A. equina*

only anemones of the red/brown colour morph were collected (the red/brown colour morph has previously been shown to exhibit higher levels of aggression than the green/orange morph – Manuel, 1988). In the lab anemones were housed individually in plastic tanks (23 x 16 x 17.5cm) containing 700ml of filtered, aerated seawater (the seawater was pumped from Mount Batten, Plymouth UK; grid reference: SX 4871 5319) and maintained at $15^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Throughout the experiment, anemones were fed *ad libitum* on aquaria marine flakes every 2-3 days and seawater was changed every 7 days.

After a 7 day acclimatisation period, anemones were dislodged from their position in the tank and provided with stones to attach to. On the afternoon of the next day, anemones were randomly paired and placed into the centre of a clean plastic tank containing 700ml of clean aerated, filtered seawater. Anemones were positioned such that their body columns were touching in order to stimulate agonistic behaviour. This contact marked the beginning of the fight and fights were considered concluded when one anemone (the loser) either: (a.) moved an approximate distance of one pedal disc away from its opponent (estimated visually) or (b.) retracted its tentacles completely for at least 10 minutes. After this point anemones were separated and returned to their tanks. At the end of the contest individuals were checked for the presence of acrorhagial peels. Individuals were then classified into the following groups: - No injuries; received injuries (i.e. peels attached to the column); inflicted injuries (i.e. holes present in acrorhagi). These groups were further broken down into winners and losers for analysis. All fights were recorded using a Canon LEGRIA HF R706 High Definition Camcorder.

All pairwise encounters that resulted in a fight were included in the analysis outlined below. Pairs that failed to fight were removed from the study.

(b) Mucus collection and lysozyme-like assays

Mucus collection and lysozyme-like assays were carried out following the methods of Stabili et al. 2015, which allows samples to be collected non-invasively and thus at several time points in the same individuals. Lysozyme-like activity (our experimental design does not allow characterization of a specific lysozyme, so cell lysis is attributed to a lysozyme-like enzyme) was measured at 3 different time points, once on the morning before the fight and

twice post-fight (1 hour and 24 hours after the fight). In order to observe how lysozyme activity varies over time in *A.equina* naturally, mucus was also collected and analysed for a subset of control anemones ($n = 23$) that did not participate in staged fights. At these time points, anemones were removed from their tanks, washed with sterile seawater and placed individually into clean dry petri dishes where they were left for 30 minutes to stimulate mucus secretion (Stabili et al., 2015). After 30 minutes, the excreted mucus was collected and centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant was then transferred into a fresh microcentrifuge tube and frozen at -20°C until use.

Lysozyme-like activity was measured using a standard intra-agar lysis assay in which 7mL of 0.04M sodium phosphate agarose (pH 6.0) was inoculated with 700µl of 5mg/mL of freeze-dried *Micrococcus lysodeikticus* (Sigma-Aldrich, Gillingham, Dorset, UK) and then spread across a Petri dish. Once dry, six wells of 6.3mm in diameter were punched into the agarose using a core borer and each filled with 30µl of mucus. The dishes were then incubated at 37°C for 22 hours, after which the diameter of the lysed area around each well was measured to the nearest 0.1mm using callipers. All samples were carried out in duplicate and an average lysis diameter calculated from the two measures.

All experimental procedures adhered to the ethical guidelines of the Association for the study of Animal Behaviour (ASAB). After use in this study all anemones were returned to the collection site at Portwrinkle. No licences or permits were required for this study.

(c) Statistical analysis

To investigate the change in lysozyme activity over time in anemones that did not fight, we conducted a repeated measures linear mixed effects model on the control anemones using the lmer function of the R package lme4 (Bates et al., 2015). We included lysozyme activity as the response variable, occasion (1, 2 and 3) as a fixed effect and anemone ID as a random intercept effect.

To test the effect of pre-fight immune response on fight outcome and injury state, we conducted two generalised linear mixed effects models (GLMMs) with a binomial error family (logit link function) using the glmer function of R package lme4 (Bates et al., 2015),

with pre-fight lysozyme-like activity as a fixed effect and fight outcome and injury state as response variables, respectively. As relative size difference (RSD) between opponents has previously been shown to impact contest dynamics in *A. equina* (Rudin and Briffa 2011), RSD was calculated (as described in Rudin and Briffa 2011) and included as a covariate in the models. To control for the effect of including both individuals from a single fight in the model, fight ID was included as a random effect. Next, to compare lysozyme-like activity at the three sampling occasions between fight outcomes (win, lose or draw) and injury states (received injuries from opponent, received no injuries or received self-inflicted injuries) we conducted two separate repeated measures linear mixed effects models (using the lmer function of the lme4 package) with lysozyme activity as the response variable and either fight outcome or injury state as a fixed effect. Occasion (pre-fight, 1 hr post-fight, 24 hrs post-fight) was included as a fixed effect in both models, along with body size as a covariate to account for any correlation between body size and lysozyme activity. Anemone ID and fight ID were included as nested random effects. To allow us to investigate the effects of receiving and inflicting injury on lysozyme activity, we conducted a further model in which only individuals that had been involved in injurious fights were included. In order to explore significant effects revealed by these models at a finer scale, we conducted post-hoc linear contrasts using the R package lsmeans (Lenth, 2016).

3. Results

(a) Change in lysozyme-like activity over time

There was a significant effect of occasion on the lysozyme-like activity of control anemones ($X^2_1 = 10.91$, $P = 0.004$). Post-hoc linear contrasts revealed that the lysozyme activity of control individuals declined significantly between occasions 1 and 3 ($P = 0.003$).

(b) Lysozyme-like activity and fighting success

Pre-fight lysozyme-like activity had no effect on the eventual fight outcome ($X^2_1 = 0.032$, $P = 0.98$). However, comparison of lysozyme activity across fight outcomes and over time

revealed a significant two-way interaction between occasion and outcome ($X^2_1 = 10.53$, $P = 0.03$). Post-hoc analyses revealed that the lysozyme activity of losers declined significantly across all three occasions (comparisons across occasions: - 1-2: $P = 0.007$; 2-3: $P = 0.03$; 1-3: $P < 0.0001$) whilst the lysozyme activity of winners only differed significantly between occasions 1 and 2 ($P = 0.004$), increasing again between measures 2 and 3. Individuals that drew showed a similar decline in lysozyme activity to losers, but to a lesser extent, with lysozyme activity only differing significantly between occasions 1 and 3 ($P = 0.04$), reflecting the temporal pattern seen in control anemones (figure 2a).

(c) Lysozyme-like activity and injury state

There was a significant effect of pre-fight lysozyme-like activity on injury state ($X^2_1 = 5.32$, $P = 0.02$) indicating that individuals that inflicted injuries during a fight expressed lower pre-fight levels of lysozyme-like activity than those that did not use their weapons. When including all fights (non-injurious and injurious) in the analysis, we found no interaction between injury state and occasion on lysozyme activity ($X^2_1 = 7.53$, $P = 0.11$). However, when we analysed only individuals involved in injurious fights, we found a significant two-way interaction between occasion and injury state ($X^2_1 = 6.06$, $P = 0.048$). Using post-hoc analyses we found that the lysozyme activity of individuals that received injuries declined significantly between occasions 1 and 3 ($P = 0.004$), whereas for individuals that inflicted injuries this post-fight decline was absent. Thus anemones that inflicted injuries during fights had lower levels of lysozyme-like activity prior to fighting and maintained these pre-fight levels after the fight (figure 2b).

4. Discussion

Immune response has previously been shown to correlate with fighting success, with winners generally exhibiting higher immunity than losers (Dijkstra et al., 2011; Filby et al., 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012). However, while these previous studies demonstrate a clear link between immunity and winning, immunity was only measured at one time point in each of these experiments (either before or after a

fight), and thus the causal direction of the relationship has so far been unclear. Does immune state affect the ability to win fights, or does immunity change as a consequence of fighting? The results of our study indicate that contrary to what has been suggested in previous work, pre-fight immunity does not predict fighting success in the beadlet sea anemone *Actinia equina*. In contrast, pre-fight immune state does appear to influence how animals will fight, specifically the decision to use weapons during a contest. Furthermore our results show that fighting itself can directly influence an individual's immune response and moreover that the extent of this effect is dependent on both fight outcome and the agonistic behaviours expressed during the fight.

We found no evidence to suggest that pre-fight lysozyme-like activity (referred to hereafter as lysozyme activity) significantly affected fighting success in *A. equina*. However, pre-fight lysozyme activity did appear to influence whether or not an individual used its weapons (acrorhagi) to inflict damage on its opponent during the fight. As aggression is linked to fighting success (in *A. equina* for example, individuals that inflict the most damage are most likely to win fights – Rudin and Briffa, 2011), and previous studies have found a positive correlation between fighting success and immunity (Dijkstra et al., 2011; Filby et al., 2010; Koskimäki et al., 2004; Rantala and Kortet, 2004; Steiger et al., 2012), we would expect to find a positive correlation between immunity and aggression, but in fact we found the opposite. Individuals that attacked their opponent exhibited lower levels of pre-fight lysozyme activity in comparison to individuals that did not. At first glance these results seem counterintuitive, suggesting that individuals that chose to express costly agonistic behaviours were in poorer condition than those that refrained from fighting injuriously. However, pre-fight lysozyme levels of those that fought non-injurious are likely to reflect a higher infection load such that these individuals were in a worse condition to begin with. This explains why these individuals then chose not to engage in agonistic behaviours that would lead to self-inflicted damage during the contest. If they are already fighting infections through elevated lysozyme activity then they would have a reduced capacity to cope with the additional costs of damaged acrorhagi.

Although pre-fight immune response did not predict fight outcome, fight outcome itself had a significant effect on post-fight immune response. Individuals of all three fight outcomes (loser, draw and winner) exhibited a significant reduction in lysozyme activity

within one hour of the fight, with individuals involved in fights with clear outcomes (win/lose) exhibiting the highest degree of reduction. However, over the next 24 hours the patterns of winners and losers began to diverge with the lysozyme activity of losers continuing to decrease while the lysozyme activity of winners increased, albeit not to pre-fight levels. This suggests that while both winners and losers suffer a similar reduction in immunity immediately after a fight, winners are able to recover from this loss quicker. These results also suggest that being involved in a fight with a clear outcome has a greater effect on post-fight immune response compared to being in a fight that ends in a draw, possibly indicating that 'clear cut' fights are more costly.

Post-fight change in immune response was also affected by injury state. Individuals that were involved in non-injurious fights as well as those that received attacks exhibited a severe reduction in lysozyme activity one hour after the fight, a pattern similar to that seen in winners and losers. This reduction continued over the next 24 hours for individuals that had received attacks but appeared to level off for those involved in non-injurious fights. Individuals that used their weapons (acrorhagi) to inflict attacks on their opponents on the other hand did not exhibit any significant change in lysozyme activity after the fight, maintaining their pre-fight lysozyme level at both post-fight time points. This result suggests that while individuals that inflicted attacks had lower lysozyme activity before the contest, they were better able to maintain this stable immune state following a fight. Differences in post-fight immune response between individuals that inflicted and individuals that received attacks might be expected due to differences in injury accrual. In *A. equina*, individuals are unable to inflict attacks on their opponent without also injuring themselves (self-inflicted damage – Lane and Briffa, 2017), ripping holes in their acrorhagi in order to leave behind necrotising peels. However, while both attackers and recipients incur injuries in *A. equina*, the type of injury accrued is not the same. Attack recipients are damaged by the application of nematocyst-filled 'peels' which cause localised necrosis while attackers are left with open wounds in their acrorhagi. Arguably we might therefore expect the risk of infection to be higher for attackers. For this reason, the ability to sustain pre-fight levels of immune response after a fight may be beneficial to attackers. However, even if the injuries received by attackers do not result in an increased susceptibility to infection, the necrosis caused by peels will still require a heightened immune response to heal. Thus, the continued reduction

in post-fight immunity seen in recipients may result in an extended healing time for the wounds incurred. However, it should be noted that we have measured only one aspect of immune response here, while the immunity of *A. equina* as with all individuals is multifaceted (Stabili et al., 2015) and thus the whole picture is likely more complex.

Taken together, our findings indicate that immunity can influence strategic decisions in a fight (to attack or not) and that fighting can significantly affect subsequent immunity in *A. equina*. Furthermore, the severity of this effect depends both on fight outcome and on the agonistic behaviours displayed during the fight. Fighting experience is known to have substantial effects on an individual's subsequent fighting success. For example, individuals that win a fight often go on to win subsequent fights, while those that lose keep on losing (winner effect and loser effect, respectively – Hsu et al., 2006; Rutte et al., 2006). Although the mechanism behind these experience effects has been debated, one suggestion was that they derive from physiological shifts or constraints induced by the first fighting experience (Hsu et al., 2006; Rutte et al., 2006). Our data show how differences in post-fight immunity, and moreover in the ability to maintain/recover pre-fight immunity levels, could go on to affect individuals' subsequent fights. If such immunological shifts are common, they may provide a mechanistic explanation for both strategic decisions and the effects of fighting experience on subsequent contests. Thus, immune status can be added to the factors already known to influence the decision to fight, including resource value (Arnott and Elwood, 2008) and morphological correlates of RHP (Lailvaux and Irschick, 2006).

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Competing interests

No competing interests declared.

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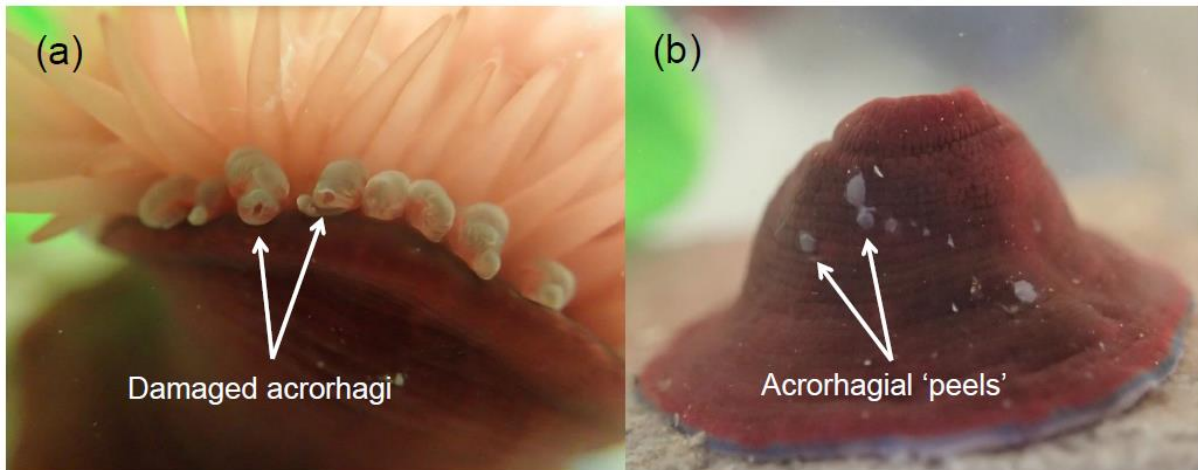
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Figure 1 (a) Damage to the attacker – holes left in acrorhagi after attack and (b) damage to the recipient – necrotising acrorhagial 'peels' left behind by attacker

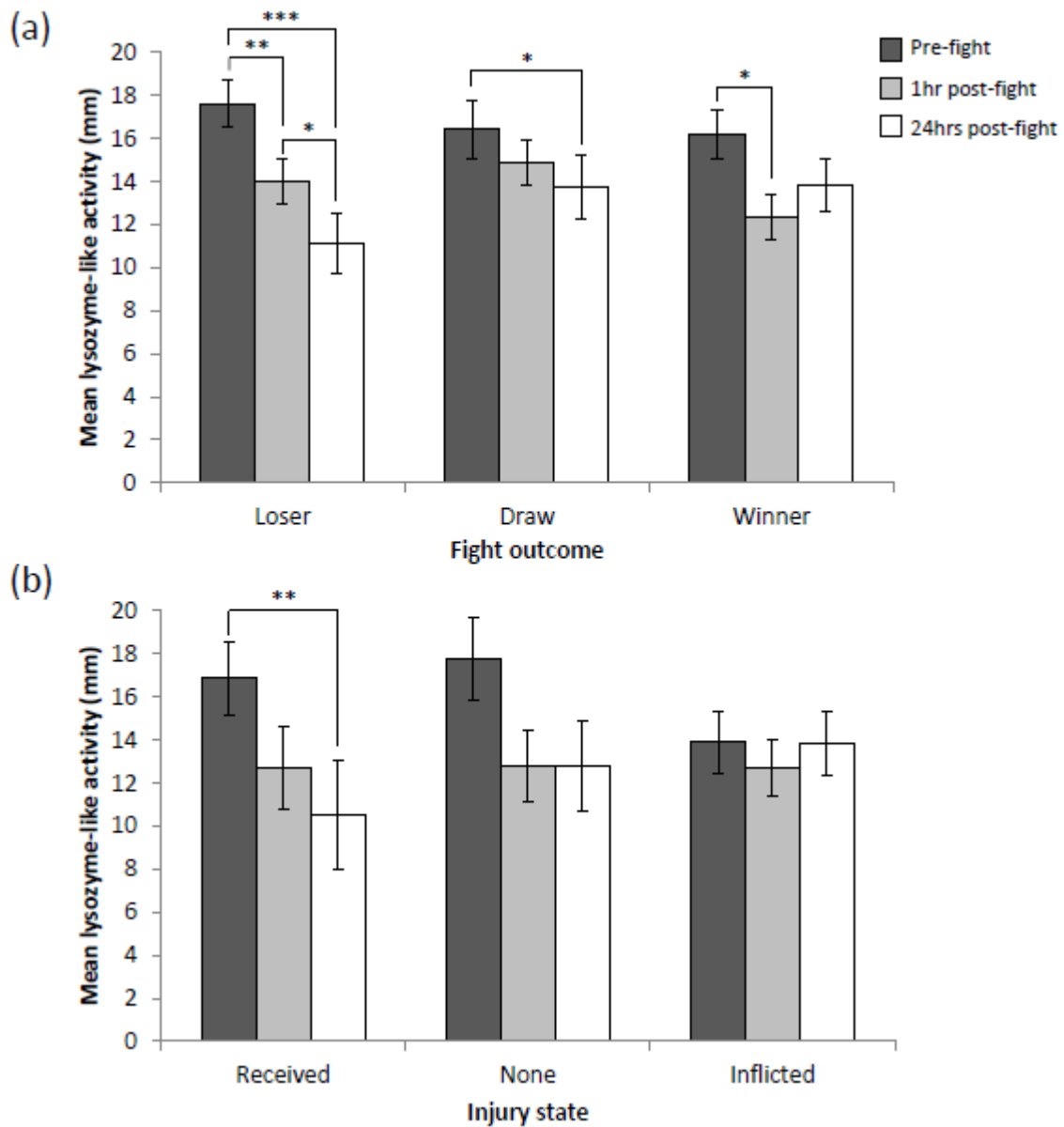


Figure 2 Average \pm SE lysozyme-like activity (mm) between (a) fight outcomes (n = loser - 25; draw - 22; winner - 26) and (b) injury states (n = received - 11; no peels - 50; inflicted - 12) across all three occasions. Asterisks indicate significant differences within groups across the three occasions as determined by least squares means (*= ≤ 0.05 ; **= ≤ 0.01 ; ***= ≤ 0.001).